

Article

pH Controlled Impedimetric Sensing of Copper(II) Ion Using Oxytocin as Recognition Element

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Received: 19 August 2018; Accepted: 11 October 2018; Published: 15 October 2018



Abstract: We report the modulation of the specific metal gation properties of a peptide and demonstrate a highly selective sensor for copper(II) ion. The neuropeptide oxytocin (OT) is reported for its high affinity towards Zn^{2+} and Cu^{2+} at physiological pH. The binding of the metal ions to OT is tuned by altering the pH of the medium. OT was self-assembled on glassy carbon electrode using surface chemistry, and electrochemical impedance spectroscopy (EIS) was used to probe the binding of Cu^{2+} . Our results clearly indicate that at pH 10.0, the binding of Cu^{2+} to OT is increased compared to that at pH 7.0, while the binding to Zn^{2+} becomes almost negligible. This proves that the selectivity of OT towards each of the ions can be regulated simply by controlling the pH of the medium and hence allows the preparation of a sensing device with selectivity to Cu^{2+} .

Keywords: oxytocin; copper(II) ion; impedimetric sensor; surface chemistry

1. Introduction

During the past few years, numerous peptide- and protein-based electrochemical sensors have been exploited for the detection of metal ions [1–6]. The selective metal ligation of peptides is derived from the specific amino acids sequence and conformations [7,8]. Oxytocin (OT) is a neuro peptide that has an affinity for metal ions and highly conserved mediator of physiological and psychic processes. Binding of OT to different divalent metal ions, notably to Zn^{2+} or Cu^{2+} , affects its interaction with the receptor, which regulates the signaling pathways either by activation or deactivation [9,10].

In our previous studies, we proved that OT can serve as a recognition element with an affinity towards both Zn^{2+} and Cu^{2+} compared to other relevant ions. However, at physiological pH, there was no selectivity between Zn and Cu and we had to use masking agents to discriminate between them [11]. OT metal binding takes place through unique mechanisms; at physiological pH (7.0), Zn^{2+} forms an octahedral complex through the OT carbonyl oxygens, while Cu^{2+} forms a square-planar complex via several deprotonated OT amide nitrogens [9]. We opted to take advantage of these differences in the binding mechanism to enable selective sensing of Cu^{2+} in the presence of Zn^{2+} . We hypothesized that the deprotonation of the OT amides at pH 10.0 will be more preferable and, hence, would lead to enhanced binding to Cu^{2+} [12]. The pH effect will also help to eliminate OT affinity towards Zn^{2+} since, at a higher pH, the formation of $Zn(OH)_2$ is more prominent and hence we turned to OT complex to see if we could use the pH of the medium for selective sensing of Cu^{2+} (Figure 1). The immobilization of vertically aligned OT onto glassy carbon electrode (GCE) was conducted using dibenzocyclooctyne as a spacer that avoids the use of Cu^{2+} for click chemistry, as previously reported [11]. Hence, it facilitates the ease of trace level detection of Cu^{2+} . In this work, we studied OT interaction with Zn and Cu at pH 10 using electrochemical impedance spectroscopy (EIS) and compared the selectivity with pH 7.0. We observed an enhanced selectivity of the proposed sensor towards Cu^{2+} in comparison to Zn^{2+} .

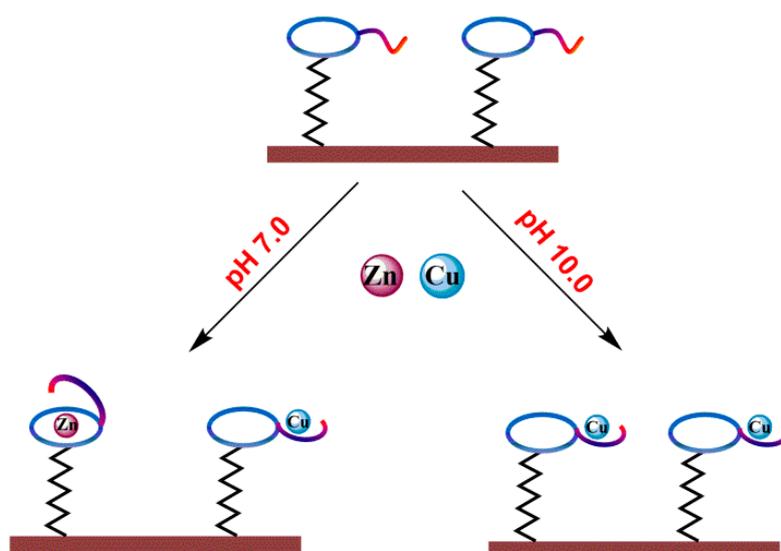


Figure 1. Tuning the binding activity of oxytocin (OT) anchored to the surface by altering the pH of the medium. OT binds both Zinc and Copper are similar affinity at physiological pH (left), while OT binds selectively Copper at alkaline pH (right).

2. Experimental Section

2.1. Material and Methods

Oxytocin, 3-aminopropyl(triethoxysilane) (APTES), dibenzocyclooctyl-*N*-hydroxysuccinimide (DBCO-NHS) and *N*-(dimethylaminopropyl)-*N*'-carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich, Rehovot, Israel. Ethanol (ACS grade), potassium hexacyanoferrate (III), potassium hexacyanoferrate (II), sodium phosphate dibasic, and sodium phosphate monobasic were purchased from Merck Chemicals (Burlington, MA, USA). All chemicals were AR grade and used without further purification. Aqueous solutions were prepared using Millipore water received from Milli-Q system (Millipore Inc., Burlington, MA, USA). The pH of the medium was adjusted to 10.0 by gradual addition of 0.1 M NaOH to phosphate buffer solution. OT functionalized with azide was synthesized by standard microwave-assisted Fmoc solid phase peptide synthesis protocol. Synthesis was performed on solid support with repeating steps of Fmoc removal and amino acids coupling. Fmoc was removed from the amine in a solution of 1:4 piperidine/*N*-methyl 2-pyrrolidone (NMP) and the next protected amino acid coupled with a solution of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) as a coupling agent, diisopropylethylamine as the base, and NMP as the solvent. The full sequence of OT cyclized by microwave-assisted disulfide formation in oxidative conditions of Iodine dissolved in 2% *v/v* anisole/NMP. Azido acetic acid coupled to the N-terminal part of the OT to give azide functionality for click chemistry. The peptide was cleaved by standard TFA cleavage procedure and purified by preparative HPLC.

2.2. UV-Visible Spectroscopy Titrations

Coordination complexes of different OT to Cu^{2+} ratio were prepared as follows. Various dilutions of Cu^{2+} solution were prepared from stock solution of 100 μM $\text{Cu}(\text{NO}_3)_2$ (99.999% trace metal basis) in phosphate buffer solution (pH 10.0). OT stock solution of 100 μM was prepared in phosphate buffer solution (pH 10.0). The stock solution was diluted to a concentration of 10 μM . Fixed OT concentration of 10 μM was added to different concentrations of Cu^{2+} ca. 10 μM , 20 μM , . . . , 80 μM . The well-mixed solutions of coordination complexes were analyzed for UV-visible absorbance spectra (Shimadzu 3101, Kyoto, Japan).

2.3. Fabrication of OT-Modified Electrode

The fabrication process of the OT Sensor was confirmed by following the physical characterization of OT immobilized on silicon wafer in the same manner as the OT Sensor [11]. The protocol uses dibenzocyclooctyne that triggers the click chemistry even in the absence of Cu^{2+} [13].

Initially, the glassy carbon electrode (GCE) surfaces were thoroughly polished using 0.05 μm alumina suspensions on micro cloth pads (CH instruments, Austin, TX, USA). Then, the electrodes were sonicated in triple distilled water for 15 min. The cleaned GCE surface was modified with the following steps. Then, the mirror finished hydroxylated GCE (GCE-OH) surface was suspended in 1% KOH solution and the solution was stirred at 100 rpm for 15 min. Approximately 4 μL of 2% APTES in AR grade ethanol was reacted with dried GCE-OH for 2 h. The electrode was washed with ethanol to remove excess APTES and byproducts, blown by nitrogen gas flow, and dried for 3 h. The amine functionalized GCE (GCE-NH₂) was incubated for 6 h in a cocktail of 0.5 mg dibenzocyclooctyne *N*-hydroxysuccinimidyl ester (DBCO-NHS) and 0.25 mg *N*-(dimethylaminopropyl)-*N'*-carbodiimidehydro chloride) in 1 mL ethanol (ACS grade). After 6 h, the electrode was removed from the solution and washed thoroughly with ethanol three times and air dried. The concentration of *N*-(2-azidoacetyl)-oxytocin (OT-AZ) was measured using Thermofisher Scientific spectrophotometer (Model: Nanodrop One/One^c) and diluted with the stock solution to get the desired concentration. GCE-DBCO was incubated for 12 h in 0.1 M phosphate buffer solution (PBS) at pH 7.0 containing 50 μM of OT-AZ. After the incubation, the OT-coated electrode was washed with PBS buffer and dried under nitrogen gas.

2.4. Electrochemical Impedance Studies

Electrochemical impedance studies were conducted using a Biologic potentiostat, model SP-300. The EIS measurements were conducted in the 3-electrode system consists of glassy carbon electrode (GCE) as working electrode and Ag/AgCl (Sat. KCl) as reference electrode and spiral platinum wire as counter electrode. All EIS measurements were carried out in room temperature at half-wave peak potential (0.210 V) of the redox mixture in phosphate buffer solution (100 mM, pH 10.0) containing a mixture of 5 mM $[\text{Fe}(\text{CN})_6]^{4-}$ and 5 mM $[\text{Fe}(\text{CN})_6]^{3-}$. The AC frequency was scanned, ranging from 100 kHz to 0.01 Hz with excitation amplitude of 10 mV. The change in charge transfer resistance (R_{CT}) at electrode/electrolyte interface was investigated using the EIS technique.

3. Results and Discussion

3.1. Job's Plot

The stoichiometric ratio of coordination complex between OT and Cu^{2+} was studied at pH 10.0 using Job's plot [14]. The method is well known for quantifying stoichiometry of host-guest molecules based on the spectral changes observed. The UV-visible absorbance spectra of the coordination complexes with different ratio of the metal ion (Cu^{2+}) were recorded. Figure 2 shows the plot between absorbance and molar fraction of the Cu^{2+} ion ($f_m = [\text{Cu}^{2+}]/([\text{OT}] + [\text{Cu}^{2+}])$). The stoichiometry of coordination complex formed was determined from Job's plot according to Equation (1):

$$n = \frac{f_{\text{peak}}}{1 - f_{\text{peak}}} \quad (1)$$

The plot (Figure 2) shows the f_{peak} value at 0.5 and, on substituting the value in Equation (1), the coordination ratio of OT- Cu^{2+} complex is 1:1. The ratio unveils that there could be only one Cu^{2+} ion coordinating with one molecule of OT. We postulated intermolecular interactions of OT to Cu^{2+} at pH 7.0, where more than one OT is involved in the coordination with one Cu^{2+} . The titrations at pH 10.0 further prove different binding mechanisms between OT and Cu^{2+} from pH 7.0.

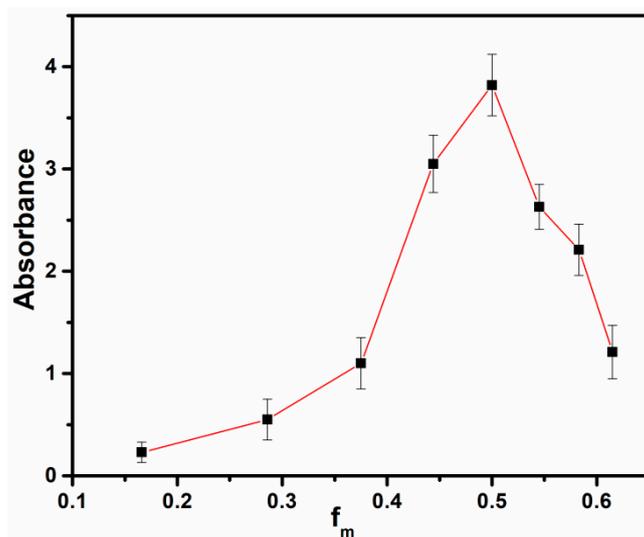


Figure 2. Jobs plot showing f_{peak} at 0.5 reveals 1:1 coordination complex between Oxytocin and Cu^{2+} . Error bars represent the standard deviation calculated from three repetitive experimental results.

3.2. Impedimetric Detection of Cu^{2+}

In our previous report [11], we reported the high affinity of OT towards Zn^{2+} and Cu^{2+} (at pH 7.0) among the various metal ions, but we could not discriminate between the two without the use of masking agents. It is proven that, although the binding mechanism for Zn and Cu is different, the binding affinity in pH 7.0 is similar. We harnessed the difference in the binding mechanism to enable selectivity toward Cu^{2+} and decided to evaluate the effect of pH on selectivity. Impedimetric sensing of Zn^{2+} and Cu^{2+} ions (1 nM) was conducted after incubating in the respective ion solution at pH 7.0 and pH 10.0 (Figure 3). The sensor signal is presented as the normalized charge transfer resistance (R_{CT}), which is calculated as the ratio between R_{CT} for the concentration of M^{2+} ($R_{\text{CT}}(\text{C}_i)$) and blank solution ($R_{\text{CT}}(\text{C}_0)$) of the sensor. Our study shows that, at pH 7.0, there is significant response from the OT sensor, both to 1 nM Zn^{2+} and to 1 nM Cu^{2+} . However, the response of OT sensor towards Cu^{2+} and Zn^{2+} at pH 10.0 is very different. At pH 10.0, no significant signal was observed in the presence of 1 nM Zn^{2+} . By contrast, the response for 1 nM Cu^{2+} was enhanced in comparison to that at pH 7.0.

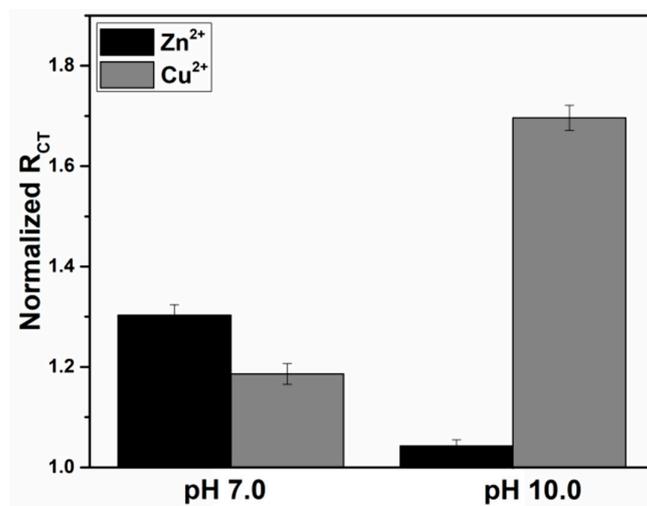


Figure 3. Histograms showing the sensor response to 1 nM Zn^{2+} and Cu^{2+} at pH 7.0 and pH 10.0. Selective detection of 1 nM Cu^{2+} at pH 10.0 and negligible response from 1 nM Zn^{2+} at the same pH.

The results provide strong evidence to support our hypothesis that metal detection is governed by the binding mechanism. Since the ligation of Zn^{2+} to OT takes place through carbonyl oxygens, while Cu^{2+} chelation of OT takes place through deprotonated nitrogens of amide, the pH plays a crucial role in the binding. At pH 10.0, the deprotonation of amides is more favorable and, hence, increases the affinity towards Cu^{2+} . We assume that the lack of affinity towards Zn^{2+} at pH 10.0 results from the preferred formation of zinc hydroxide and the conformational changes associated with the electrostatic repulsions of the deprotonated amides.

After the confirmation of selective detection of Cu^{2+} at pH 10.0 using the OT sensor, the sensor response towards various Cu^{2+} concentrations was studied. The OT sensor was exposed to increasing concentrations of Cu^{2+} , ranging from 1 pM to 0.1 μ M, and the corresponding impedance spectra were recorded. The obtained Nyquist plots are shown in Figure 4A. The gradual increase in the diameter of the semicircle with the increase in Cu^{2+} concentration represents an increase in the R_{CT} attributed to the metal ion binding. Figure 4B presents the logarithmic concentration of Cu^{2+} versus normalized R_{CT} . The sensor shows the linear correlation between the R_{CT} and Cu^{2+} over a wide range of concentrations—1 pM to 0.1 μ M—with an enhanced sensitivity of $0.16 M^{-1}$ in comparison to that at pH 7.0 [11]. We assume that high pH facilitates the deprotonation of amide nitrogens and makes coordination bonding between OT and Cu^{2+} stronger. The detection limit of the OT sensor for Cu^{2+} sensing was determined according to the formula: $LOD = [(3.3 \times S.D) - a]/b$, where S.D is the standard deviation of the blank, a is the linear coefficient, and b is the sensitivity. The calculated LOD is found to be $2.65 \times 10^{-13} M$.

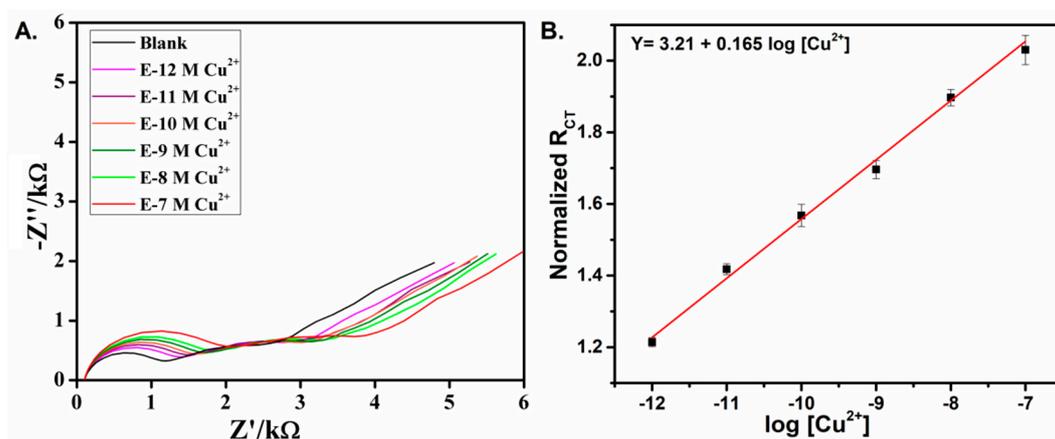


Figure 4. (A) Nyquist plots obtained for that OT Sensor in 5 mM $[Fe(CN)_6]^{3-/4-}$ consists of 0.1 M phosphate buffer solution (PBS) at pH 10.0 after incubation in various Cu^{2+} concentrations and (B) logarithmic concentration of Cu^{2+} vs. normalized charge transfer resistance (R_{CT}).

4. Conclusions

Peptides are natural metal chelators and serve as attractive sensing elements. The unique combination of functional groups and conformational flexibility determines the peptide's metal binding mechanism. We showed here that we can harness the differences in binding mechanisms of OT toward metal ions to achieve enhanced selectivity. We relied on the deprotonation-assisted OT–Cu binding mechanism and showed that using a high pH in the sensing media significantly increases the affinity of the sensor toward copper compared to using pH 7.0. Furthermore, we proved that we can selectively determine Cu^{2+} in the presence of Zn^{2+} at pH 10.0 without using masking agents. This study suggests that relying on the unique mechanisms of peptide metal interactions can be used for enhancing sensitivity and increase the selectivity of sensors by rational design. We believe that this approach might be very important for other peptide-based sensors for metal ions and other analytes.

Author Contributions: K.K.T., M.H., and S.Y. conceived and designed the experiments; I.A. synthesized the azido-OT; K.K.T. and I.A. performed the experiments and all the authors discussed the work; K.K.T. wrote the paper. M.H. and S.Y. supervised the work.

Funding: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 664786.

Acknowledgments: The authors would like to thank RECORD-IT project. S.Y. would like to thank the the Benjamin H. Birstein Chair in Chemistry.

Conflicts of Interest: The authors declare no conflict of interest.

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